Demonstration of inter- and intraspecies differences in serotonin binding sites by antibodies from an autistic child

(autoantibodies/serotonin receptors/autism)

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ABSTRACT Serotonin (5-HT) binding sites from bovine and rat cerebral cortex membranes share pharmacological properties that allow both to be subclassified by the same criteria. We show here that [3H]5-HT binding sites from human cortex also possess pharmacological properties that follow the same subclassification scheme as for bovine and rat cortex. In addition, we show that solubilized 5-HT1 and 5-HT3 sites from all three species have an \( \text{B}_{	ext{max}} \) value of 3.4. Despite these similar pharmacological and physical characteristics, we can demonstrate antigenic differences between receptor types and species. Human 5-HT1 sites can be distinguished from human 5-HT1, 5-HT2, and 5-HT3 sites and from equivalent sites in rat and bovine cortex. The anti-human 5-HT1A antibodies were discovered in the blood of an autistic child and may have clinical or etiologic significance for this disorder.

Brain serotonin (5-HT) binding sites have been classified into several categories. Peroutka and Snyder (1) and Pedigo et al. (2) have divided rat cortical [3H]5-HT binding sites into three types. 5-HT1A sites have nanomolar affinity for 5-HT and spiperone and micromolar affinity for ketanserin. 5-HT1B sites possess nanomolar affinity for 5-HT and micromolar affinity for spiperone and ketanserin. 5-HT2 binding sites have micromolar affinity for 5-HT and nanomolar affinity for spiperone and ketanserin. Recent evidence from our laboratory (3) and from Fillion et al. (4) suggests the existence of a fourth type of 5-HT binding site. This moiety is present in bovine cortex and is a [3H]5-HT binding protein with 50–250 nM affinity for [3H]5-HT but micromolar affinities for spiperone and ketanserin-like compounds (refs. 3 and 4; unpublished data). In an effort to maintain consistency in nomenclature, we will refer to this last site as a 5-HT3 type. We have also solubilized the 5-HT1 and 5-HT3 sites from bovine cortex and described their biophysical characteristics (3).

In the course of a study on hyperserotonemia in autistic children, we found a child with circulating antibodies directed against human cortical 5-HT binding sites. Further investigation disclosed that these antibodies cross-reacted only with the 5-HT1A subclass of 5-HT1 binding sites. This gave us the opportunity to study antigenic properties of the 5-HT1A class of binding sites across species. In this report, we compare the properties of 5-HT1 and 5-HT3 sites from human, rat, and bovine cortex, describe the characterization of the antibodies from the autistic child, and demonstrate intra- and interspecies differences in antigenicity in 5-HT binding sites.

MATERIALS AND METHODS

The child reported here is a 9-year-old girl who was in good health and free of all medications at the time of the study. She met DSM-III (5) criteria for infantile autism, as determined by three independent, experienced clinical evaluators. Venous blood samples were collected into EDTA-containing tubes. Platelet 5-HT as measured by the method of Stahl et al. (6) was 72 ng per 10⁶ platelets or 550 ng/ml of whole blood. IgG class antibodies were purified from serum samples by protein A-Sepharose 4B (Sigma) affinity chromatography (7). Greater than 85% of serum IgG was recovered in one column passage. Human frontal cortex tissue was obtained at autopsy. Membranes from human, bovine, or rat cortex were prepared as described (3). Gel electrophoresis was performed on 10% acrylamide/0.1% sodium dodecyl sulfate slab gels. Coomassie blue staining and densitometry were carried out as described (8). High molecular weight standards (Sigma) were electrophoresed for molecular weight determinations. The agaro IgG fraction of goat anti-human IgG was obtained from Miles–Yeda (Rehovot, Isra- el).

Samples were analyzed for [3H]5-HT binding by equilibrium saturation (Scatchard) analysis (9) as described (3), except as follows: membrane samples were incubated with and without purified antibodies for 20 min at 30°C and then placed on ice for 2 hr. The equivalent serum dilution was 1:3 in all cases. [3H]5-HT (0.5–200 nM, 28 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and 10 μM pargyline were then added to aliquots of membranes containing 80 μg of protein, with or without added IgG, in duplicate and incubated for a further 20 min at 30°C. Samples were then placed on ice, filtered, and assayed for radioactivity as described (3). Specifically bound radioactivity was determined by the addition of 10 or 100 μM 5-HT to equivalent assay tubes (3). Saturation curves were resolved into two components by computer analysis and separate affinity constants \((K_a)\) and site concentrations \((B_{max})\) were determined (3). Minimal antibody titers were calculated by determining the absolute reduction in \(B_{max}\) of a particular binding site per ml of serum. This probably underestimates the actual titer of antireceptor antibody since only antibodies that block [3H]5-HT binding are detected.

A sample of cerebrospinal fluid (CSF) was obtained from a lumbar puncture of the child done as part of another study (consent of parents obtained). CSF was dialyzed against 50 mM Tris-HCl (pH 7.4) and used directly in assays or pre-treated with agarose-linked goat anti-human IgG antibody to remove human IgG.

5-HT1 and 5-HT3 receptors were solubilized as described elsewhere, except that 3% Triton X-100 and 1% Tween-80 were used (3). Sedimentation coefficients \((s_{20,w})\) were estimated by comparison with the sedimentation of bovine serum albumin \((s_{20,w} = 4.6)\) on 10–30% glycerol gradients containing detergent (3).

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Abbreviations: 5-HT, serotonin; CSF, cerebrospinal fluid.

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RESULTS

Pharmacological and Physical Similarities in [3H]Serotonin Binding Sites. As discussed above, sufficient pharmacological similarities exist across species to apply the same subclassification system to [3H]5-HT binding proteins from rat (1, 2) and bovine (refs. 3 and 4; unpublished data) cortex. We have found that the same classification scheme holds for human frontal cortex as well. Fig. 1 shows a direct comparison of [3H]5-HT saturation binding (Scatchard analysis) for frontal cortex membranes from all three species. In the rat, there is a single component representing binding to 5-HT1 receptors. The addition of 1 μM spiperone significantly reduces the number of observed sites, as described (2). The reduction in [3H]5-HT binding in the presence of spiperone defines the 5-HT1A (spiperone sensitive) and 5-HT1B (spiperone insensitive) sites in rat.

The [3H]5-HT binding curves for bovine and human cortex membranes, however, are distinctly different. Human and bovine cortical membranes have the same high-affinity 5-HT1 component as is present in rat membranes. In contrast, they also possess a lower affinity component. This has been described in bovine cortex (refs. 3 and 4; unpublished data). The lower affinity component is referred to here as a 5-HT3 site.

Addition of 1 μM spiperone decreases binding to the 5-HT1 component of bovine and human membranes. These membranes thus show the same distinction between 5-HT1A and 5-HT1B sites as exists in rat membranes (2). However, the relative proportions of the 5-HT1A and 5-HT1B sites differ between species. The percentage of 5-HT1A sites for the three species is human, 48% ± 5%; bovine, 16% ± 4%; and rat, 30% ± 5% (duplicate determinations). This compares well to previous reported values for rat cortex membranes (2). A more extensive pharmacological characterization of human frontal cortex [3H]5-HT binding sites will be reported elsewhere.

As discussed above, the pharmacological characteristics of the different types of [3H]5-HT binding proteins appear the same in cortical tissue from rat (1, 2), bovine (refs. 3 and 4; unpublished data), or human origin. We have shown previously that bovine 5-HT1A, 5-HT1B, and 5-HT3 binding proteins can be solubilized by a combination of Triton X-100 and Tween-80 detergents (ref. 3; unpublished data). We also found that these solubilized species comigrated on Sephadryl S-300 column chromatography and glycerol velocity sedimentation gradients (3). This suggests that these [3H]5-HT binding proteins have similar sizes and shapes.

It became of interest to extend these findings to human and rat cortex. Fig. 2 shows that the sedimentation coefficients and profiles for [3H]5-HT binding sites solubilized from all three species are the same. This suggests that not only the pharmacological characteristics but also the physical characteristics of the 5-HT1 class are conserved across species. The 5-HT3 site is not present in the rat (refs. 1 and 2 and Fig. 1).

Antigenic Differences in [3H]5-HT Binding Sites. Infantile autism is a pervasive developmental disorder of early life (5), which is frequently associated with hyperserotonemia (10). In spite of intensive study, no adequate explanation for the hyperserotonemia has been found (11, 12). We hypothesized that serotonergic dysfunction in these children might be secondary to immune phenomena directed against serotonin receptors.

During a screening for autoimmune phenomena in children afflicted with infantile autism, we discovered a child with circulating antibodies that inhibited [3H]5-HT binding to human frontal cortex membranes. We have been able to characterize these antibodies and use them to study 5-HT receptor subtypes.

Since serum contains several additional components that bind [3H]5-HT with low affinity, we separated IgG class 1, 2, and 4 antibodies from other serum proteins by protein A-agarose affinity chromatography (7). This procedure isolates IgG proteins with high yield (>85%) and purity (>98% as judged by gel electrophoresis). Pretreatment of the isolated IgGs by agarose-linked goat anti-human IgG eliminated >98% of all protein material (data not shown).

Fig. 3 shows the effects of added IgGs from the autistic girl on [3H]5-HT binding to human cortical membranes. In the absence of purified antibodies, the binding isotherm shows a distinct biphasic curve with affinity constants (Kds) of about 1 and 250 nM (Fig. 3A, closed circles). As described above, the 1 nM component is due to a combination of 5-HT1A and 5-HT1B sites, whereas the 250 nM component represents binding to 5-HT3 sites. Preincubation of membranes without isolated IgGs did not change the binding isotherm. However, when membranes are preincubated with IgGs...
from the autistic girl, there is a decrease in the 5-HT$_1$ component of the Scatchard curve (Fig. 3A, open circles). This decreased binding reflects a reduction of the number of binding sites ($B_{max}$) but not a change in affinity ($K_d$). Thus, preincubation of membranes with IgG from the autistic child decreased the number of 5-HT$_1$ sites available for $[^3]$H$5$-HT binding.

To determine whether this child’s IgGs recognized 5-HT$_{1B}$ sites, the above experiment was repeated in the presence of 1 $\mu$M spiperone, which can be used to differentiate $[^3]$H$5$-HT binding to 5-HT$_{1A}$ and 5-HT$_{1B}$ sites. When this was done, no detectable change in 5-HT$_{1B}$ sites was seen. Fig. 3B shows the effect of preincubation of membranes with or without the autistic child’s IgG, which were then assayed for $[^3]$H$5$-HT binding in the presence of 1 $\mu$M spiperone. There is no significant difference between the two curves. These data suggest that the antibodies from this child block $[^3]$H$5$-HT binding only to spiperone-sensitive 5-HT$_{1A}$ sites.

It also was of interest to determine whether anti-5-HT$_{1A}$ receptor antibodies were present in the CSF of the autistic child. Preincubation of membranes with dialyzed CSF from this child caused a reduction in the apparent $B_{max}$ of the 5-HT$_{1}$ component (Fig. 4A). Pretreatment of the CSF with goat anti-human IgG antibody-linked Sepharose blocked this reduction, showing that the effect is IgG-mediated in CSF as well (Fig. 4B). The inhibitory component concentration per ml was 4-fold higher in this sample than in a serum sample drawn at the same time (data not shown).

Since the antibodies from the autistic child distinguished human 5-HT$_{1A}$ sites from human 5-HT$_{1B}$ and 5-HT$_{3}$ sites, it was of interest to determine whether antigenic similarities also existed between species. Membranes were incubated with and without added IgG and then assayed for $[^3]$H$5$-HT binding. As shown in Fig. 5, no IgG-mediated inhibition of $[^3]$H$5$-HT binding was observed for bovine or rat cortex membranes. This suggests that despite the pharmacological and physical similarities among 5-HT$_{1A}$ sites in the three species, differences exist in antigenic domains such that antibodies directed against one species do not cross-react with equivalent domains from the others. The antigenic domains recognized by this child’s antibodies may represent the $[^3]$H$5$-HT binding site of the human 5-HT$_{1A}$ receptor itself or other areas that, when occupied by antibody, cause conformational changes in the $[^3]$H$5$-HT binding site. There may be extensive antigenic cross-reactivity between the spe-
cies in other antigenic domains that would not have been detected by the assay method used here.

**DISCUSSION**

The results in this report describe the discovery of autoantibodies against 5-HT binding proteins present in the blood and CSF of an autistic child. These antibodies are of the IgG class, discriminate between 5-HT1A and other 5-HT binding proteins, and are specific for human 5-HT1A sites. Use of them, even in the limited way described here, has provided helpful insights into structural similarities and differences among 5-HT binding proteins.

Antibodies against nicotinic cholinergic receptors have played a major role in elucidating the structure of these proteins, their subunit relationships, and their position in cell membranes (see ref. 14 for review). In addition, there appears to be at least one clinical disorder in humans, myasthenia gravis, in which autoantibodies exist against nicotinic receptors (13).

The characterization and classification of other neurotransmitter receptors have been difficult and at times confusing. Pharmacologic characterization has been widely employed to differentiate neurotransmitter receptors, but the applicability of this procedure is limited by the lack of specificity of most available ligands. Antibodies directed against various domains on these molecules would immensely facilitate their characterization. However, with the exception of the β-adrenergic receptor, for which an antibody has recently been prepared (15), and the nicotinic receptor described above, no other neurotransmitter receptor has been sufficiently well purified to permit preparation of antireceptor antibodies by routine methods. Hopefully, the discovery of other anti-5-HT receptor antibodies will expand our understanding of neurotransmitter system.

What is the relationship of these findings to the etiology, symptoms, or course of infantile autism? Clearly, an observation on just one child demands cautious interpretation. The finding of a 4-fold higher CSF antibody titer, however, suggests that our observation may be clinically meaningful and related to reports of hyperserotonemia in autistic children (10). Since infantile autism is not a single disease entity, but rather a syndrome caused by diverse etiologies (11, 12, 16), it is tempting to speculate that a subgroup of autistic patients may be suffering from an autoimmune disorder. We have now undertaken a larger study of autistic, mentally retarded, and normal children to test the validity of this initial observation. Of 13 autistic children so far studied, 7 have circulating antibodies directed against human brain 5-HT receptors. In contrast, these antibodies were not seen in 13
normal children (unpublished data).

The observed degree of inhibition of [3H]5-HT binding and the serum dilutions necessary to achieve it (about 1:3) may call the clinical significance of these findings into question. We decided, therefore, to calculate the absolute titer of detectable antibodies that blocked [3H]5-HT binding and compare this with a known autoimmune disorder, myasthenia gravis. Patients with this disease have circulating antibodies against motor end-plate nicotinic receptors and suffer a variety of deficits in skeletal muscle function. In their original report on serum globulin binding to acetylcholine receptors, Almon et al. (13) used an assay similar to the one described here, which measured the inhibition of [125I]-α-bungarotoxin binding to acetylcholine receptors by sera from patients with myasthenia gravis. Using their stated values for receptor concentrations, we calculated the maximal observed antibody titer in their patients to be about 45 fmol/ml of serum. By comparison, the observed antibody titers for this autistic child were 600 and 980 fmol/ml of serum for two samples drawn 1 month apart. The level of antibody present in this child, therefore, could well be clinically significant.

The antibodies of the child in this report directly discriminate 5-HT1A binding proteins from 5-HT1B, 5-HT2, and 5-HT3 binding proteins present in the same tissue. This is consistent with the known differences in pharmacological profiles for these proteins (refs. 1–3; unpublished data). As stated before, differences detected are in antigenic domains, which, when occupied by antibody, block [3H]5-HT binding, either sterically or by inducing conformational changes in the [3H]5-HT binding site. Whether structural differences also exist in other portions of these proteins is unknown. Also, whether antibodies directed against other portions of the human 5-HT1A protein are present in this child is unknown.

The present results further demonstrate antigenic differences between human, bovine, and rat 5-HT1A binding sites. As shown here, the pharmacological (refs. 1–3; unpublished data; Fig. 1) and physical (ref. 3; unpublished data; Fig. 2) characteristics of these sites are similar. Presumably, some as yet undetermined structural differences exist. The functional significance of these differences remains to be shown. It is conceivable that the antigenic sites recognized by this child's antibodies are directed against associated membrane components that are species specific and that the binding proteins themselves are identical. The testing of this hypothesis awaits purification and further biochemical characterization of 5-HT binding sites from all three species.

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